AD_____

Award Number: W81XWH-08-1-0058

TITLE: Survival Signaling in Prostate Cancer: Role of Androgen Receptor and Integrins in Regulating Survival

PRINCIPAL INVESTIGATOR: Laura Lamb

CONTRACTING ORGANIZATION: Van Andel Research Institute
Grand Rapids, MI 49503

REPORT DATE: January 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 Jan 2009	Annual Summary	1 Jan 2008 – 31 Dec 2008

4. TITLE AND SUBTITLE

Survival Signaling in Prostate Cancer: Role of Androgen Receptor and Integrins in Regulating Survival

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0058

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Laura Lamb

E-Mail: laura.lamb@vai.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Van Andel Research Institute
Grand Rapids, MI 49503

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Although prostate cancer patients initially respond to androgen ablation therapy, they ultimately relapse and the tumor no longer responds to androgen, offering little hope for long-term disease-free survival. However, inhibition of AR expression in cells leads to cell death. This suggests that prostate cancer cells are still dependent on AR for survival, even if the cells are no longer responding to physiological levels of androgen. We have demonstrated that expression of AR in PC3 prostate tumor cells can rescue cells from death induced by inhibition of PI-3K. AR expression leads to increased expression of α6β1integrin and subsequent increased expression of the pro-survival protein Bcl-xL. Blocking either leads to cell death. We have also generated an in vitro differentiation model of the prostate epithelium which generates differentiated AR-expressing secretory cells that recapitulate many in vivo characteristics. Using this model, we have demonstrated that prostate secretory cells are dependent on PI-3K and E-cadherin, but not EGFR, integrins, or androgen, signaling for cell survival.

15. SUBJECT TERMS
Cell biology, integrins, androgen receptor (AR), signal transduction, survival signaling, differentiation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

INTRODUCTION

Prostate cancer is the second leading cause of cancer death in men and death is due to metastasis. While primary prostate tumors are often curable, metastatic tumors are not. Androgen ablation therapy has been the most commonly prescribed treatment for metastatic prostate cancer for the last sixty years. Androgen ablation therapy prevents androgen function by inhibiting both the production of androgen and its binding to its receptor, androgen receptor (AR). Although patients initially respond to treatment, they ultimately relapse and the tumor no longer responds to androgen, offering little hope for long-term disease-free survival. However, inhibition of AR expression or its DNA binding activity even in androgen independent (i.e. non-responsive) cells inhibits their proliferation and leads to cell death. This suggests that prostate cancer cells are still dependent on AR for survival, even if the cells are no longer responding to physiological levels of androgen. Thus targeting AR directly or its downstream effectors that regulate survival would be a more effective therapeutic approach for targeting and killing prostate cancer cells. Development of new strategies for more effective treatment of prostate cancer is limited by an incomplete understanding of the mechanisms regulating cell survival of either normal prostate or prostate cancer cells.

Prostate cancer arises from the epithelial layer of the prostate. The normal prostate epithelium consists of two types of cells, basal cells and secretory cells. In the basal cells, which do not express AR, adhesion to the extracellular matrix in the basement membrane is required for cell survival. In the secretory cells, which do express AR, survival is independent of matrix and is suggested to be regulated by AR since these cells die during androgen ablation therapy. In normal prostate epithelial, adhesion to matrix and AR expression are mutually exclusive events. However, in prostate cancer, the tumor cells express AR and are adherent to matrix, allowing for interactions between these two signaling pathways. **My hypothesis was that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival, while AR regulates survival of normal cells independently of integrins.** The objective of these studies is to identify the AR- and integrin-mediated mechanisms which regulate survival in AR expressing tumor and normal prostate cells. By understanding the activities that lie downstream of AR that directly regulate survival of the tumor cells versus normal cells, a specific approach to disrupt AR-dependent actions only in the tumor cells can be developed, which will lead to the death of tumor cells without harming normal prostate tissues.

BODY

My working hypothesis is that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival, while survival of normal cells is regulated independently of integrins. To accomplish the tasks outlined in the statement of work, AR expressing prostate cancer cells and AR expressing normal cells first had to be generated as previously described and validated in the original submission. We have also optimized siRNA to AR in order to verify AR-dependent effects.

Summary of Aim 1:

The goal of Aim 1 in our Statement of Work was to determine how AR signaling mediates survival in prostate cancer cells *in vitro*. My *working hypothesis* was that AR activation will independently regulate the same downstream survival targets as those regulated by the PI-3K/Akt pathway, such as survivin, such that inhibition of signaling from either PI-3K/Akt or AR can be rescued by the other pathway**.** I proposed to have the tasks in Aim 1 completed in the first year of funding. **Task A** was to determine if AR expression affects integrin-mediated survival signaling pathways in DU145s. **Task B** was to determine if LM

signaling via AR to survivin rescues survival in PI-3K inhibited cells. Emphasis was placed on the **Task B** and Aim 2, and given time constraints, this leaves **Task A** still needing to be accomplished. However, the **Task B** is nearly complete. Treatment of PC3 cells with LY294002, which blocks PI-3K signaling, results in cell death (1). In accordance with my preliminary data, treatment of PC3-AR-1 or PC3-AR-2 cells adherent to LM1 with LY294002 failed induce cell death as measured by trypan blue staining, propidium iodide (PI) staining for sub G1 cells, or Terminal Deoxynucleotide Transferase dUTP Nick End Labeling (TUNEL) staining (**Fig. 1 A-C**). This was independent of androgen (DHT), the physiological ligand for AR. This difference in survival was not strictly due to cell cycle status since PC3-AR-1 cells grow faster and PC3-AR-2 cells grow slower than PC3-puro cells (**Fig. 1 D**). To verify that this effect was due to AR expression and not clonal selection, I collaborated with another graduate student in the lab, Jelani Zarif, to knock down AR expression in the AR expressing cells using siRNA prior to treatment with the PI-3K inhibitor, LY294002 (**Fig. 1 E**). We demonstrated that loss of AR in AR expressing clones increased cell death when the cells were treated with LY294002 (**Fig. 1 F**). Overall, this data suggests that AR is a pro-survival factor that acts independently of PI-3K signaling and DHT.

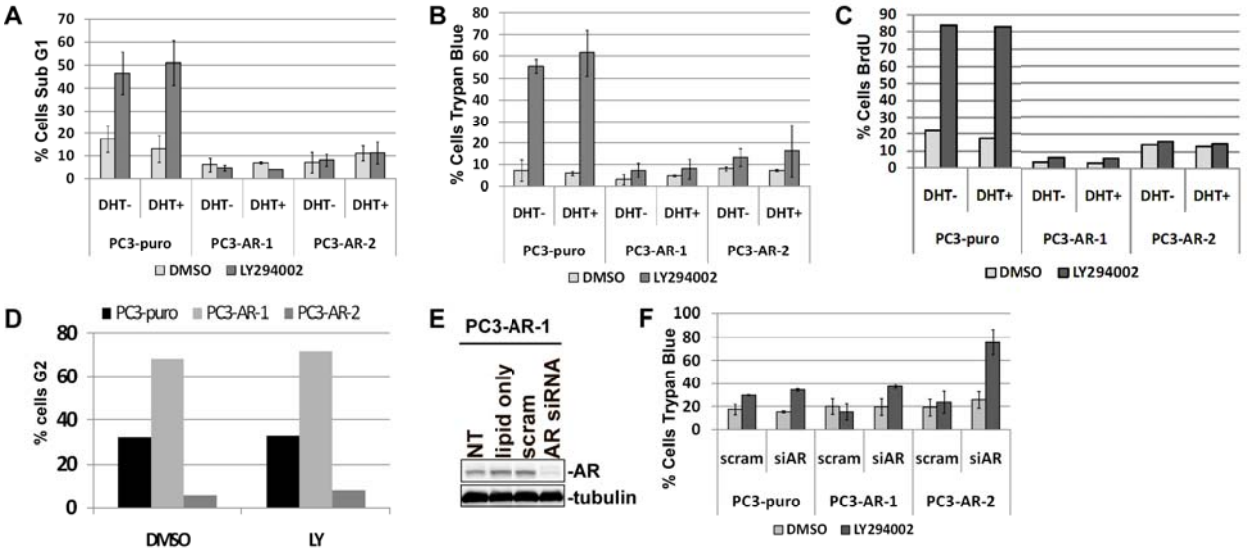

Figure 1: AR promotes survival independent of PI-3K and androgen. Cells were plated on LM1 and treated with DHT and DMSO or LY492004 (LY). After 72hr cells were either fixed, permeabilized, and stained with propidium iodide (PI) to detect (**A**) dead cells (sub G1) and (**D**) cell cycle status (shown as cells in G2 Phase) , (**B**) counted for trypan blue staining, or (**C**) fixed and permeabilized for nicked DNA using TUNEL staining. (**E**) PC3-AR-1 cells were treated with nothing (no treatment), siLentFect lipid (Lipid only), nonspecific siRNA (scram) or AR siRNA. The levels of AR expression were monitored by immunoblotting. Similar results were obtained for PC3-AR-2 (data not shown). **(F)** Cells were treated with siRNA against AR (siAR) or a non-specific sequence (scram) 72h prior to plating on LM1 and treated with DMSO or LY294002. Cell viability was assessed 72h after inhibitor addition by trypan blue staining. Error bars on all graphs represent standard deviations; n=3-4 (n=2 for C). (*A-E) Laura Lamb, (F) Jelani Zarif*

Since AR expression lead to an increase in integrin α6, and epithelial cells are dependent on signaling from the matrix through integrins for survival (2, 3), I hypothesized that AR was promoting survival through up-regulation of integrin α6. To test this, integrin α6 expression in the AR expressing clones was decreased back to empty vector levels by careful titration of integrin α6 siRNA (**Fig. 2 A**). Non-specific siRNA (scram) was used as a control. Cells were treated with integrin α6 siRNA or scram for 72 hours to induce partial knock-down of integrin α6, then cells were plated on LM1 and treated with LY294002. After 72 hours, cell viability was assessed by trypan blue staining. A 63-73% reduction in α6 integrin expression in

the presence of LY294002 is sufficient to induce cell death in AR expressing cells (**Fig. 2 B**). These data indicate that AR is promoting survival through integrin α6.

A

	siRNA	
Cell Line	Scram	ITGA6
PC3-puro	20.42	5.32
PC3-AR-1	61.28	23.15
PC3-AR-2	59.01	16.09

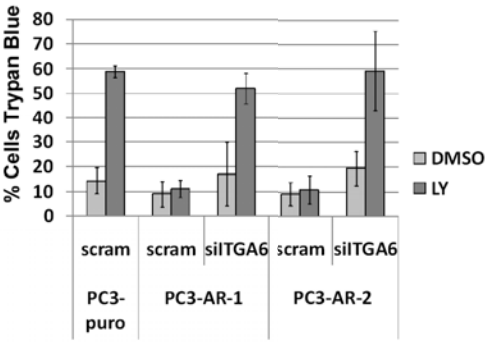

Figure 2. AR promotes survival through up-regulation of integrin α6. PC3-puro, PC3-AR-1, and PC3-AR-2 cells were treated with siRNA against 6 integrin (siITGA6) or non-specific sequence (scram) for 72 hours. (**A**) Cells were treated with florescent-conjugated antibodies against integrin α6 and analyzed by FACS. Rat IgG were negative controls. Values given are for mean florescent values minus IgG controls. (**B**) Cells were plated on LM1 and treated with DMSO or LY294002 for 72 hours. Cell viability was determined using trypan blue staining.

In **Task B**, my *hypothesis* was that AR is independently regulating survivin through crosstalk with LM integrins. Survivin can prevent apoptosis by either directly or indirectly inhibiting caspase activity (4). Treatment of LNCaP AR positive tumor cells with DHT, but not the AR negative tumor cell lines PC3 and DU145, increases expression of survivin (5). Adhesion of PC3 tumor cells to FN also increased survivin levels (6). Thus both integrins and AR can regulate survivin. However, in my AR expressing PC3 clones, there was no increase in survivin levels compared to empty vector control cells, independent of DHT or plating the cells on LM or CL matrix (data not shown). Furthermore, treatment of cells with the PI-3K inhibitor LY294002 also had no effect on survivin levels (data not shown). Together, this data suggests that in AR expressing PC3 cells, survivin is not the downstream target of integrin- and AR-signaling. Since it did not appear that survivin is the mechanism by which AR is promoting survival independent of PI-3K signaling, I did not generate virus expressing shRNA to survivin, or use the shRNA to knockdown survivin levels in AR expressing PC3 cells to determine the effects on survival.

However, I found that the pro-survival protein Bcl-xL is up-regulated in AR expressing PC3 cells (**Fig. 3 A**), which suggested that Bcl-xL could be the mechanism by which AR could promote survival independent of PI-3K signaling. To demonstrate that up-regulation of Bcl-xL is due to AR expression, AR expressing cells were treated with AR siRNA and expression of Bcl-xL was monitored. In collaboration with Jelani Zarif, we demonstrated that loss of AR in AR expressing clones resulted in down-regulation of Bcl-xL (**Fig. 3 B**). To determine if Bcl-xL expression is dependent on integrin α6, integrin α6 expression was decreased using integrin α6 siRNA. Decreased integrin α6 resulted in decreased Bcl-xL (**Fig. 3 C**). Together, these data indicate that AR promotes survival through up-regulation of integrin α6. Increased integrin α6 signaling in turn drives up-regulation of the pro-survival protein Bcl-xL.

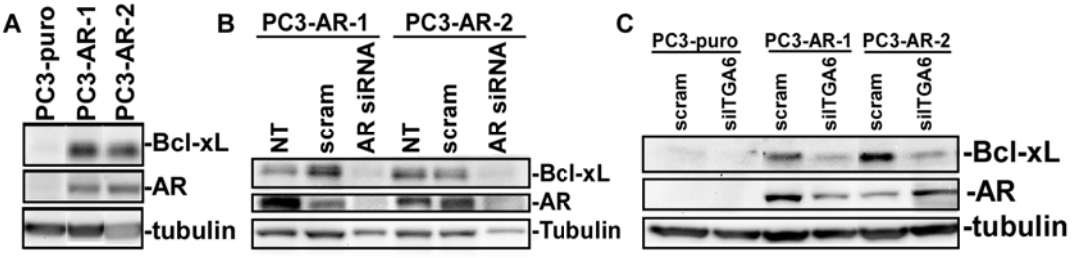

Figure 3. Up-regulation of integrin α6 leads to increased Bcl-xL expression. PC3-puro, PC3-AR-1, and PC3-AR-2 cells were treated

with siRNA against integrin α6 (siITGA6), AR, or non-specific sequence (scram) for 72 hours. Bcl-xL and AR levels were monitored by immunoblotting of whole cell extracts using Bcl-xL and AR specific antibodies. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. *(A, C) Laura Lamb, (B) Jelani Zarif*

To determine if Bcl-xL is required for PI-3K-independnet survival, Bcl-xL was knocked down in the AR expressing cells to the levels found in the PC3-empty control cells using Bcl-xL siRNA. Partial loss of Bcl-xL in the AR expressing clones in the presence of LY294002 induced cell death to the levels found in the PC3-empty cells treated with LY294002 (**Fig. 4 B**). Complete loss of Bcl-xL resulted in complete loss of viability (data not shown). To demonstrate that Bcl-xL over-expression is sufficient to promote survival independent of PI-3K signaling, retroviruses were used to infect cells with an empty vector or a vector to express Bcl-xL,and stable clonal cell lines were selected. Bcl-xL over-expression to the levels found in AR expressing clones was confirmed by immunoblotting (**Fig. 4 C**). Bcl-xL over-expressing cells did not die when treated with LY294002 (**Fig. 4 D**). Bcl-xl overexpression did not result in AR expression or changes in integrin expression (**Fig. 4 C**, data not shown).This data suggests that Bcl-xL can promote survival independent of PI-3K signaling, and that AR is promoting survival by up-regulation of Bcl-xL.

Expression of AR in PC3 cells did not result in hyper-activation of the PI-3K/Akt pathway compared to empty vector control cells (data not shown). Furthermore, LY294002 was still a potent inhibitor of PI-3K/Akt signaling in AR expressing cells (data not shown). Downstream pro-survival targets of PI-3K signaling, such as inhibition of the pro-death protein Bad by phosphorylation and increased expression of the pro-survival protein survivin, were unaltered in AR expressing PC3 cells compared to empty vector control cells (data not shown).

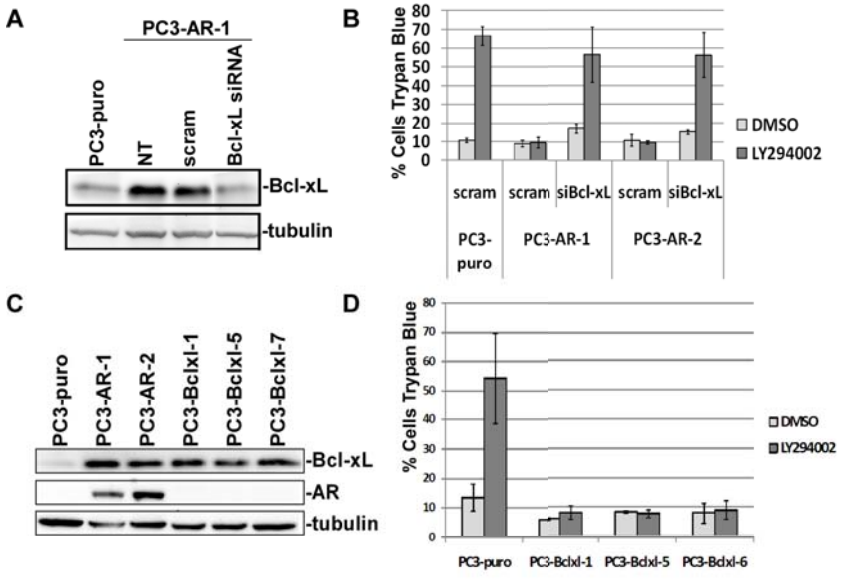

Figure 4. Bcl-xL promotes survival independent of PI-3K signaling. (**A**) Cells were treated with siRNA specific against Bcl-xL or non-specific sequence (scram) for 72h . Bcl-xL levels were monitored by immunoblotting of whole cell extracts using Bcl-xL and AR specific antibodies. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. (**B**) Cells were then treated with DMSO or LY294002 to inhibit PI-3K signaling. Cell viability was measured 72h after drug addition by trypan blue staining. (**C**) PC3 cells were made to over express Bcl-xL by infecting cells with a retrovirus containing an empty or Bcl-xL construct (PC3-puro and PC3-Bclxl respectively) and stable clones were selected in puromycin. (**D**) PC3-puro and PC3-Bcl-xl clones were plated on LM1 and treated with DMSO or LY294002 for 72h. Cell viability was then measured by trypan blue staining.

In PC3 cells, LM1 matrix induced signaling promoted Bcl-xL expression through Src activation (Fig. 1) (1). There was increased Src expression and activity in the AR expressing clones (**Fig. 5 A**). In collaboration with Jelani Zarif we demonstrated that while loss of AR expression in these cells results in partial loss of Src activity it had no effect on Src expression (**Fig. 5 B**). To test whether integrin α6 signaling was enhancing Src activity, integrin α6 expression was knocked down to PC3-puro levels using integrin α6 siRNA. Partial loss of integrin α6 failed to affect Src activity, suggesting that AR-enhanced Src activity was not downstream of integrin α6 (data not shown). In PC3-AR clones, partial knock down of Src to levels found in PC3-puro

cells does not lead to decreased levels of Bcl-xL , AR, or integrin α6 (data not shown). These data indicate that AR expression alters Src expression and activity, but it is not involved in the α6 integrin/Bcl-xL signaling axis. I still need to determine whether Src is important for PI-3K-independent survival in AR expressing cells.

I have not yet been able to generate stable PC3 cell lines expressing mutant variants of AR due to difficulty with the site-directed mutagenesis since the regions of interest were in GC-rich areas of the DNA. Instead of trying to make the DNA mutations myself, I have acquired the

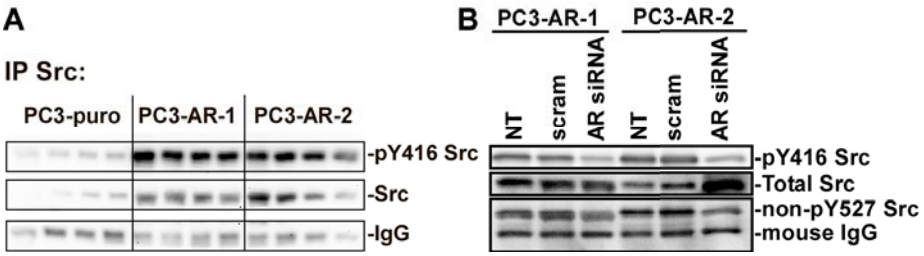

Figure 5. AR regulates Src activity. Src was immunoprecipitated (IP) and activity was measured by immunoblotting for phosphorylation of tyrosine 416 (pY416) and loss of phosphorylation on tyrosine 527 (Y527). (B) PC3-AR clones were treated with AR siRNA or a non-specific sequence (scram) for 144h, then Src activity was measured as in (A). *(A) Laura Lamb, (B) Jelani Zarif*

DNA from Dr. Charles Sawyers and Dr. Scott Dehm and have verified with sequencing that the DNA has the correct mutations. I have made lentivirus which express these AR mutants, have infected PC3 cells, and I am currently establishing stable clones. After I establish stable clones, I will test the ability of the AR mutants to rescue cell death after treatment with PI-3K inhibitor LY294002 and adhesion to LM by TUNEL staining and FACS analysis.

Additional Work: Lastly, I observed that the AR expressing clones have a different morphology and display more filapodia than the PC3-puro cells (**Fig. 6 A, B**). Correspondingly, the AR expressing clones are more migratory than the PC3-puro cells as determined by a Boyden chamber migration assay (**Fig. 6 C, D**). This preliminary data suggests a role of AR in promoting increased cell migration.

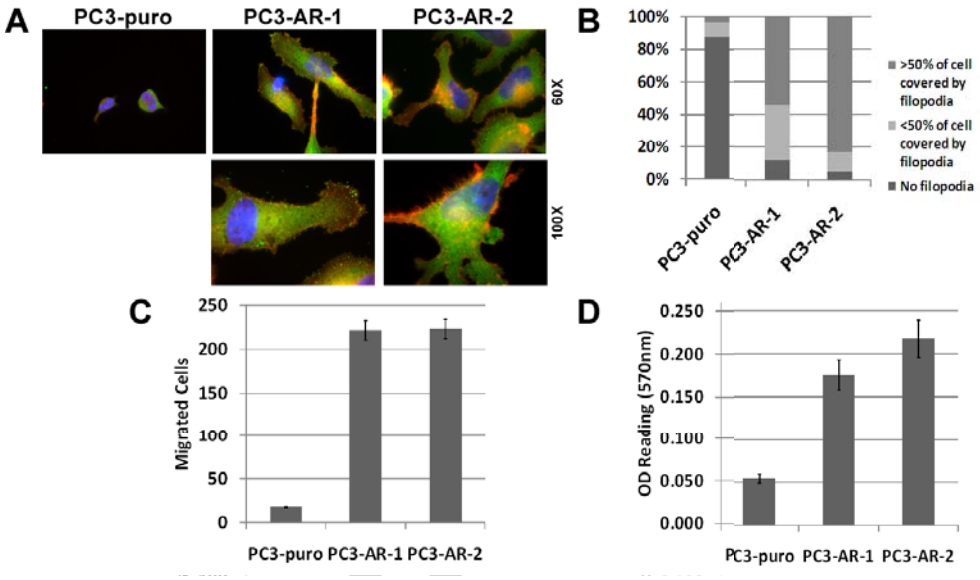

Figure 6. AR promotes filopodia formation and migration. (**A**) Cells were plated on LM1 for 1hr then fixed, permeabilized, and stained to visualize actin (red), focal adhesions (green specks), and nuclei (blue). (**B**) Quantification of the presence of filopodia on cells. 100 cells were counted per experiment; data is mean of four experiments. (**C, D**) The ability of cells to migrate was tested using a Boyden-chamber migration assay with LMI gradient as the chemoattractant. Cells that migrated to the bottom chamber were then (**C**) counted or (**D**) crystal violet staining was eluted from cells and quantified by OD reading. N=3.

In **summary** of Aim 1, I have demonstrated that AR can promote prostate tumor cell survival independent of the PI-3K pathway (**Fig. 7**). AR does not affect survivin expression. AR regulates integrin expression by decreasing integrins α3, α5, β1, and β4 and increasing integrin α6. Up-regulation of integrin α6 drives increased expression of the pro-survival protein Bcl-xL. Bcl-xL promotes survival independent of PI-3K signaling. AR also enhances Src activity, independent of integrin α6. Enhanced Src activity is not responsible for elevated Bcl-xL levels. Since the AR expressing clones promote survival and integrin changes independent of androgen, this system may provide a model for androgen-independence in metastatic prostate cancer. Within the next year, I expect to complete these studies and publish the results in a peer-reviewed journal.

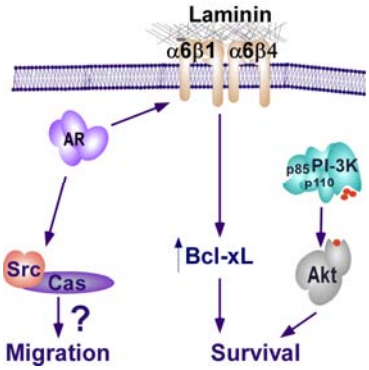

Figure 7. Model for AR signaling in PC3 cells. AR promotes cell survival independent of DHT or PI-3K signaling. AR regulates survival via the integrin α6 and subsequently Bcl-xL. AR increases Src activity independent of integrin α6. Src activity may regulate migration and survival.

Summary of Aim 2:

The goal of Aim 2 is to determine how AR mediates survival in normal primary prostate epithelial cells *in vitro.* My *working hypothesis* was that the integrin-mediated survival pathway in primary prostate epithelial cells will shift from being dependent on EGFR to being dependent on AR. In addition, AR regulates survival by directly regulating survivin. The **first task** was to determine whether AR expression affects integrin-mediated survival signaling pathways in PECs. The **second task** was to determine if LM signaling via AR to survivin is responsible for cell survival in PECs. The **third task** was to determine **i**f integrins mediate survival in PECs expressing AR. I had proposed to complete the tasks in the second to third year of funding. I have made significant progress on Aim 2 however in the first year of funding. We are currently preparing a manuscript for submission to a peer-reviewed journal describing the results I have obtained thus far and have described below.

Currently, there is no cell culture model for human secretory cells that express AR. Before I could accomplish any of the tasks for Aim 2, I first had to establish an *in vitro* model which recapitulates the biology of the epithelium of the human prostate. A recent study demonstrated that treatment with KGF can induce AR expression in prostate epithelial cells in culture (7). When I first submitted my proposal to the DOD, I had found that KGF and androgen treatment of confluent basal PEC cells over a two week period induces the formation of two cells layers. The bottom cell layer does not express AR or its target prostate specific antigen (PSA) typical of normal basal epithelial cells, whereas the cells on the top layer do – similar to secretory epithelial cells. Furthermore, while expression of AR in the top cells is uniform, expression of PSA, which is a secreted protein, is polarized with the heaviest expression being at the top side of the cells facing the medium suggesting that it is being secreted. This approach appeared to be a promising model to study AR positive secretory cells of the prostate by inducing the differentiation of basal cells in culture.

Expression of AR *in vivo* correlates with down-regulation of integrins, so next I sought to determine if integrin expression was changing in AR-expressing PEC cells. My hypothesis was that there would be a loss of all integrins except integrin α6 since this is what is reported *in vivo* and in PC3 cells expressing AR. Analysis with epifluorescent and confocal imaging revealed that the top layer cells and the cells immediately below them lost expression of all integrins including α6β1 (**Fig. 8 B, C, Table 1**). Cultured basal PECs secrete and organize a laminin 5 (LM5)-rich matrix (8); the differentiating cell population that lost integrin expression also no longer produced LM5 matrix (**Fig. 8 B,C**). FACS analysis revealed that 15-20% of the total cell

population) and integrin α6 positive cells (basal cells) (data not shown). Treatment of differentiated cultures with dissociation buffer preferentially dislodges the top layer of cells. FACS analysis indicates that 97.19 (-/+1.76)% of the isolated dislodged population is negative

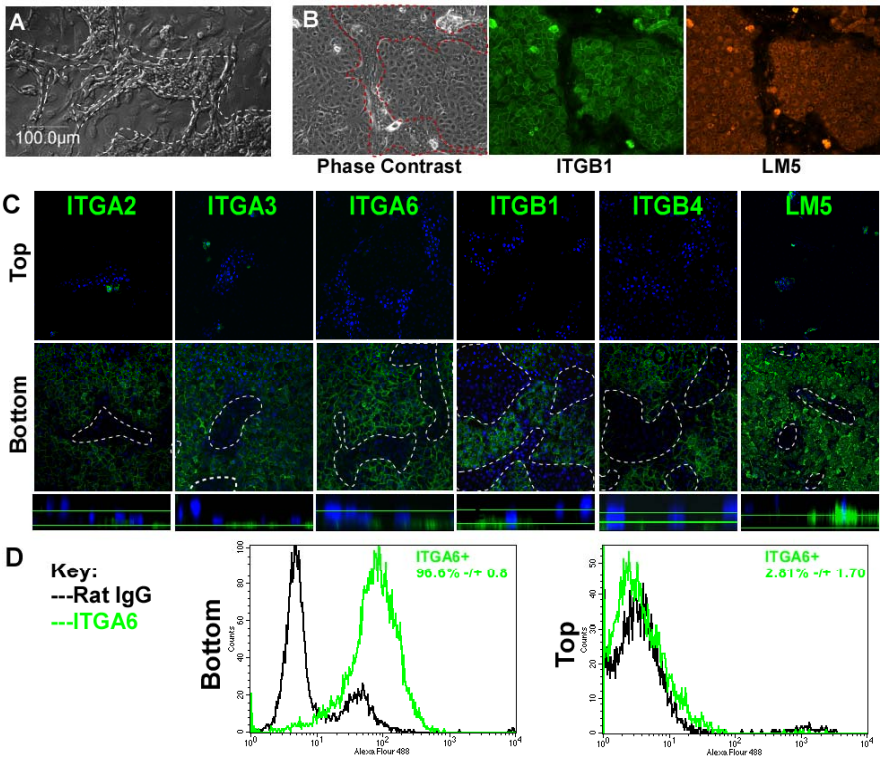

Figure 8. Primary prostate epithelial cells (PECs) were induced to differentiate with KGF and DHT treatment for 10-14 days resulting loss of integrin (ITG) and lamimin (LM) 5 expression in top cells. **A**) DIC image of differentiated culture shows a top layer of cells (outlined with a dashed white line) on top of a confluent bottom cell layer. X20. **B**) Integrin β1 (ITGB1) and LM5 expression by epifluorescent microscopy. 10 day differentiated culture was stained for ITGB1 (*green*), LM5 (*red*), and DNA (*blue*). X20. Top layer outlined in red. **C**) Expression of integrins α2 (ITGA2), α3 (ITGA3), α6 (ITGA6), α1 (ITGB1), β4 (ITGB4) and LM5. 14 day differentiated cultures were stained for indicated integrins or LM5 (*green*) and DNA (*blue*). *Top, Bottom* Representative confocal x-y sections of top layer and bottom layer cells respectively. Area located directly below top layer indicated with dashed white line. *Z* Representative z-section image complied from 10-15 confocal x-y sections representing a thickness of 17.04 (+/- 3.27) μm. Green lines indicate top and bottom of bottom cell layer. X20. **E**) FACS histograms for integrin α6 and rat IgG control expression of bottom or top cells of differentiated culture separated by cell disassociation buffer.

for cell surface α6 integrin, while 96.6 (-/+0.8)% of the cells not dislodged by dissociation buffer are still positive for α6 integrin (**Fig. 8 D**). I have been able to compare signaling and protein expression differences between the two layers by using this isolation method followed by lysing the cells and immunoblotting (data not shown). Since there is a population of bottom cells which does not express integrin α6 but may not yet express secretory differentiation markers, I am exploring using additional markers to enhance sorting for AR positive cells only. After sorting, I will use IF staining for differentiation markers to verify the cell populations' identity and purity.

To ensure that the differentiation model is a true representation of normal prostate secretory cells, I then sought to establish that AR was functional and that both cell layers were expressing markers exclusive to the different epithelial cell layers *in vivo*. The AR positive cells in the top layer, but not the basal cells, express the AR regulated proteins PSA, TMPRSS2, Nkx3.1, and PSMA (**Fig. 9 A**, some data not shown). Furthermore, these proteins were only expressed when the ligand for AR, androgen, was present (**Fig. 9 F, G**, some data not shown). Intriguingly, secretory specific markers, cytokeratins K19 and K18, were also only expressed in the presence of androgen (**Fig. 9 A,F**, K18 data not shown). KLK2, another AR-regulated marker, could be detected by RT-PCR (**Fig. 9 E**). The AR positive top layer was also negative for the basal marker Bcl-2, p63, and K5 (**Fig. 9 A**, some data not shown), while the basal cells were positive. AR expression was also detectable by immunoblotting (**Fig. 9 D**). Together, this data suggests that AR is functional and regulates differentiation; the bottom and top layers

express the same markers as basal and secretory cells do in vivo. A summary of the markers analyzed and comparison of our in vitro results with those in vivo are presented in **Tables 1 and 2**. Hereafter when referring to this model, the AR-expressing top cells will be referred to as secretory-like cells and the AR-negative bottom cells as basal cells.

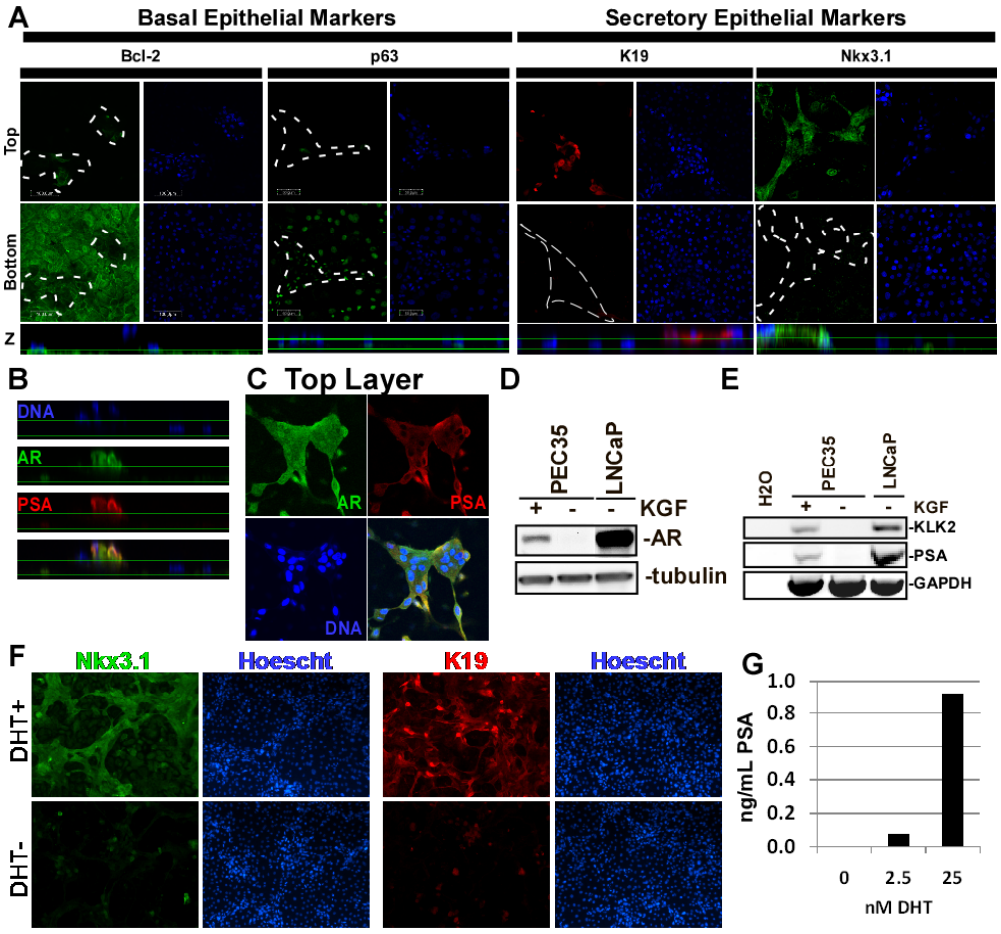

Figure 9. Differentiation Markers. Confluent PEC cells plated on CL1 were treated with KGF and DHT for 10-15 days, resulting in two cell layers. To determine if cells were undergoing differentiation, IF staining was performed for markers specific to prostate basal or secretory cells. (**A**) Representative confocal x-y sections of top layer (*Top)* and bottom layer (*Bottom*) cells respectively. Area located directly below top layer indicated with dashed white line. Representative z-section image (*Z*) complied from 10-15 confocal x-y sections representing a thickness of 17.04 (+/- 3.27) μm. Green lines indicate top and bottom of bottom cell layer. Indicated markers were stained for in green. Nuclei were stained with Hoescht (blue). 20X. (**B, C**) Confocal z-section of both layers or z-y section of top layer cells (**B**) stained for AR (green), PSA (red) and DNA (blue). X20,X60. (**D**) Immunoblotting for AR in PEC cells treated with or without KGF. LNCaP cells were used as a positive control for AR expression. Total levels of protein in the lysates were monitored by immunoblotting with anti-tubulin. (**E**) RT-PCR for KLK2, PSA, and GAPDH in PEC cells treated with or without KGF. (**F**) Epifluorescent x-y images for PEC cells treated with or without DHT. IF was performed to visualize Nkx3.1 (green), K19 (red) or nuclei (blue). (**G**) PSA ELISA for differentiated cells treated with increasing amounts of DHT.

Using this differentiation system as my model, I next wanted to determine the role of EGFR and PI-3K signaling in survival of the secretory-like cells, as described in **Task A**. To distinguish between cell death occurring in basal cells from that occurring in secretory cells in the mixed culture model, cell viability was assessed in each population using TUNEL or caspase 3 staining and confocal. Treatment of the differentiated cells with the PI-3K inhibitor LY294002 for 72 hours resulted in a 5.5 to 5.75 fold increase in TUNEL positivity only in the upper secretory cells and not the lower basal cells (**Fig. 10 B-D**). Cultures treated with the EGFR inhibitor PD198393 did not have significant TUNEL staining over DMSO (vehicle) control (**Fig. 10 B-D**). Furthermore, the ligand for AR, androgen, was unable to promote survival independent of PI-3K signaling, suggesting that AR does not promote survival of secretory cells (**Fig. 10 C, D**). It has been suggested that survival in secretory cells may be dependent on

stromal derived growth factors, such as KGF (9). The growth factor KGF was also not able to rescue survival in the presence of the PI-3K inhibitor (**Fig. 10 E**). KGF has been reported to activate p38 signaling, and Jnk signaling can promote survival during stress (7, 10). Inhibition of Erk, p38, or Jnk pathways with the inhibitors PD98059, SB202190, and 420119 respectively, did not result in significant cell death (**Fig. 10 G**), suggesting that survival was being regulated through another mechanism. I have not yet determined if Src is required for survival in secretory-like cells.

Table 1. Epithelial Markers

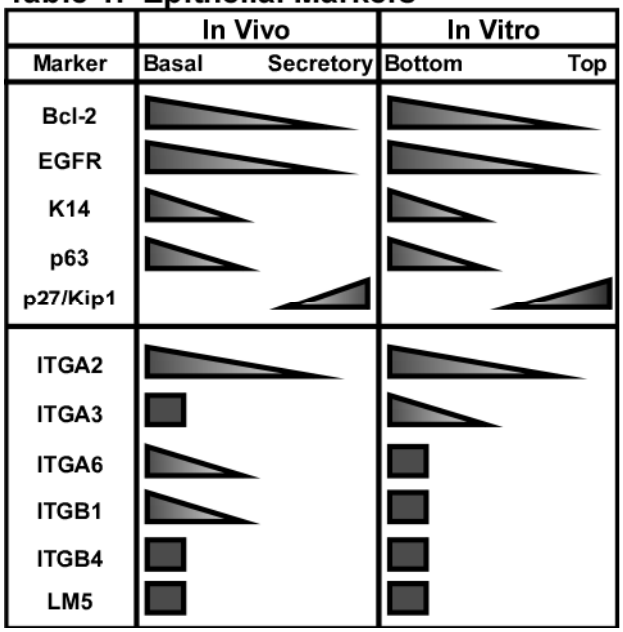

Table 2. Androgen-Regulated Markers

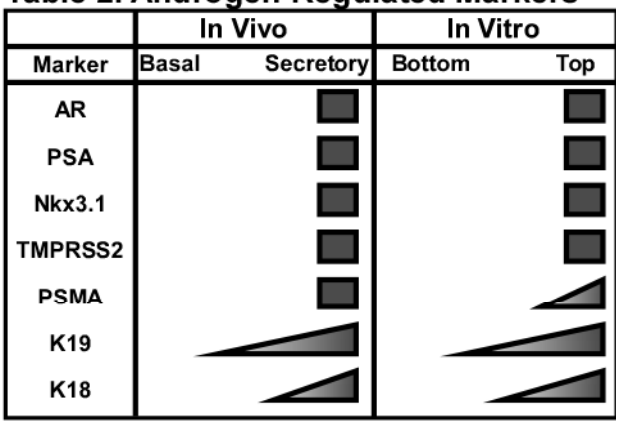

Table 1 and 2. Comparison of *In Vivo* Basal and Secretory Cell Marker Distribution to Observed Distribution in Top and Bottom Layers of *In Vitro* Differentiation Model.

The goal of **Task B** is to determine if signaling via AR to survivin is responsible for survival in secretory-like cells. Since androgen was unable to rescue cell survival in the presence of the PI-3K inhibitor, it is unlikely that AR is functioning to promote survival in secretory-like cells. I have optimized siRNA knock-down of AR in the differentiated cells. To determine the role of AR in survival, I will treat cells with non-specific sequence or AR siRNA in the presence of the PI-3K inhibitor LY294002 and monitor cell viability with TUNEL immunostaining daily up to 96 hours. Since it does not appear that AR is important for survival, survivin may not be the mechanism by which the cells are surviving. I will monitor survivin expression as outlined in my SOW. If I determine that survivin expression is not the mechanism for cell survival, then I would look at Bad activity. Secretory-like cells are dependent on PI-3K whose downstream target is Akt (**Fig. 10**); phosphorylation of Bad on Ser136 has been shown to be Akt dependent and acts to sequester Bad to protein 14-3-3, making Bad unavailable to drive apoptosis (Zha et al., 1996).

Task C is to determine if integrins mediate survival in PECs expressing AR. I have determined that the AR expressing secretory-like cells do not express integrins (**Fig. 8**), therefore integrins cannot be the mechanism by which secretory-like cells are surviving. During keratinocyte differentiation, basal cells lose integrin expression as well as adhesion to matrix as they are extruded to the upper layers of the skin. In suprabasal keratinocytes, cell-cell adhesion structures such as E-cadherin appear to promote survival through PI-3K signaling, and when PI-3K signaling is lost these cells die (11). To determine in E-cadherin was promoting survival in secretory-like cells, differentiated cells were treated with an E-cadherin blocking antibody or IgG control for 72 hours and then cell viability was assessed by TUNEL staining. Inhibition of E-

cadherin resulted in a 4 to 5 fold increase in cell death in the secretory-like cells (**Fig. 10 F**). Thus, E-cadherin is required for survival of secretory-like cells.

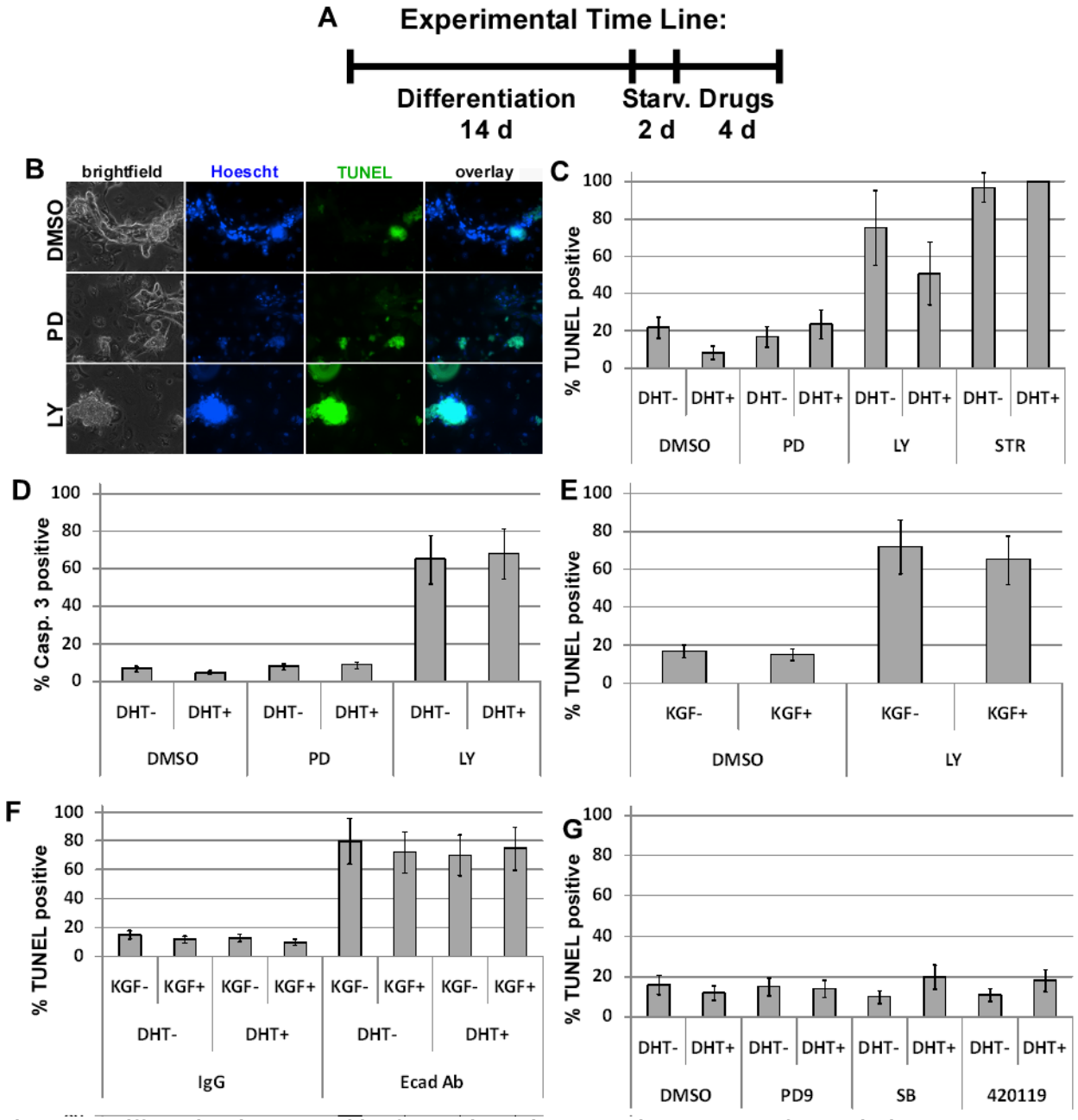

Figure 10. Differentiated PECs positive for AR depend on PI-3K, but not EGFR, for survival. PECs were treated with KGF and DHT for 14 days, starved for 2 days of growth factors, and then treated with DMSO, PD198393 (PD), and LY492004 (LY) in the presence or absence of DHT. After 72 hours of drug treatment, cell viability was accessed using TUNEL staining. Ten fields per experiment and condition were quantified for TUNEL positive green pixels and DNA positive blue pixels using the software program Imagine. TUNEL positive pixels were normalized to DNA positive pixels.

KEY RESEARCH ACCOMPLISHMENTS

1. Determined that survivin levels were not affected by adhesion to different matrices, androgen, AR expression, or treatment with the PI-3K inhibitor LY294002 in AR expressing PC3 cells.
2. Demonstrated that expression of AR in PC3 cells regulates survival via integrin α6/Bcl-xL signaling.
3. Demonstrated that expression of AR in PC3 cells up-regulates Src signaling independent of integrin α6.
4. Demonstrated that AR expression in PC3 cells results in altered cell morphology including increased filopodia expression and increases cell migration.
5. Generated and characterized an *in vitro* differentiation model of the prostate epithelium which generates differentiated AR-expressing secretory cells that recapitulate many *in vivo* characteristics.
6. Demonstrated that secretory-like cells are dependent on E-cadherin and PI-3K signaling, but not androgen, integrins, or EGFR signaling, for survival

REPORTABLE OUTCOMES

The following items have been generated due to the research carried out in the last year.

1. In the past year abstracts were presented at scientific meetings as poster presentations. A copy of one of the abstracts is included in the appendix.

   Lamb, L.E., Zarif, J.C., Miranti, C.K. 2008. Integrin Regulation of Survival in Prostate Tumor Cells Expressing Androgen Receptor. Gordon Research Conference: "Signaling by Adhesion Receptors", South Hadley, MA, June 29-July 4.

   Lamb, L.E., Zarif, J.C., Miranti, C.K. 2008. Integrin Regulation of Survival in Prostate Tumor Cells Expressing Androgen Receptor. Gordon Graduate Research Seminar: "Signaling by Adhesion Receptors", South Hadley, MA, June 28-29.

   Lamb, L.E., Zarif, J.C., Miranti, C.K. 2008. AR Regulates Bcl-xL and Integrins in Prostate Tumor Cells Expressing Androgen Receptor. Michigan Prostate Research Colloquium: "Frontiers in Urologic Cancer: Molecular Mechanisms and Therapeutic Strategies", Grand Rapids, MI, May 2-3.

2. I was elected to be on the Cell and Molecular Biology Executive Committee as the student representative.

CONCLUSIONS

Prior to these studies the role of androgen receptor signaling in survival in prostate tumor cells and normal cells was largely unknown. These studies demonstrate that re-expression of wild type AR in PC3 cells prevented the cell death normally induced upon inhibition of PI-3K signaling. Rescue of cell death occurred independently of androgen. Expression of AR in PC3 cells lead to increased expression of the pro-survival protein Bcl-x_L and α6β1integrin, and down regulation of other integrins. Loss of AR, integrin α6, or Bcl-x_L re-sensitized AR-expressing PC3 cells to PI-3K-dependent survival. The AR-induced increase in α6 integrin is responsible for the elevated Bcl-x_L levels. Thus AR regulates cell survival through

enhancement of α6β1 expression, which up-regulates Bcl-x_L, independently of PI-3K signaling. We are currently exploring the mechanism by which AR enhances α6β1 expression. We have also generated an *in vitro* differentiation model of AR positive secretory cells. Using pharmacological inhibitors we have demonstrated that in contrast to basal cells, the differentiated secretory cells are not dependent on EGFR signaling for survival, but depend on PI-3K and E-cadherin. Furthermore, androgen, the physiological ligand for AR, is unable to promote survival when PI-3K signaling or E-cadherin is inhibited.

So What: Our AR expressing PC3 cells have altered integrin expression and are able to survive independent of androgen, suggesting that these cells may provide an *in vitro* model of androgen-independent prostate tumor cells. Furthermore, these cells survive independent of PI-3K signaling, and loss of AR, integrin α6, or Bcl-xL in combination with inhibition of PI-3K signaling results in cells death. Since over 90% of metastatic prostate cancer expresses AR, inhibition of AR or PI-3K signaling alone is insufficient to induce cell death of prostate cancer cells. Rather it may be necessary to inhibit AR or a downstream target (i.e. integrin α6 or Bcl-xL) in combination with a PI-3K inhibitor for effective prostate cancer therapy. Also, I have developed a differentiation model that allows investigation of survival signaling pathways of secretory-like cells *in vitro*. I have found that AR expression correlates with loss of integrin expression in normal cells. Traditionally it has been thought that androgen and AR promote survival in prostate secretory cells, since only the secretory cells die during androgen ablation (12, 13). However, in our studies, secretory cell survival was not dependent on the presence of androgen. The lack of dependence on androgen for secretory cell survival in our human culture system is in agreement with genetic and tissue recombination studies in mice (14). Secretory cells may die during androgen ablation therapy due to loss of androgen-dependent paracrine factor secreted from the prostate stroma. Secretory-like cells were dependent on E-cadherin and PI-3K signaling for cell survival, which is similar to differentiated keratinocytes. This suggests that an underlying survival mechanism regulates survival in differentiated epithelial tissues, and androgens primary function in the normal prostate is to drive differentiation.

REFERENCES

1. Edick MJ, Tesfay L, Lamb LE, Knudsen BS, & Miranti CK (2007) Inhibition of Integrin-mediated Crosstalk with Epidermal Growth Factor Receptor/Erk or Src Signaling Pathways in Autophagic Prostate Epithelial Cells Induces Caspase-independent Death. *Mol. Biol. Cell* 18(7):2481-2490.
2. Calderwood DA, Shattil SJ, & Ginsberg MH (2000) Integrins and Actin Filaments: Reciprocal Regulation of Cell Adhesion and Signaling. *J. Biol. Chem.* 275(30):22607-22610.
3. Frisch SM & Screaton RA (2001) Anoikis mechanisms. *Curr Opin Cell Biol* 13(5):555-562.
4. Altieri DC (2001) The molecular basis and potential role of survivin in cancer diagnosis and therapy. *Trends in Molecular Medicine* 7(12):542.
5. Zhang M, Latham DE, Delaney MA, & Chakravarti A (2005) Survivin mediates resistance to antiandrogen therapy in prostate cancer. 24(15):2474.
6. Fornaro M*, et al.* (2003) Fibronectin Protects Prostate Cancer Cells from Tumor Necrosis Factor-{alpha}-induced Apoptosis via the AKT/Survivin Pathway. *J. Biol. Chem.* 278(50):50402-50411.
7. Heer R, Collins AT, Robson CN, Shenton BK, & Leung HY (2006) KGF suppresses {alpha}2{beta}1 integrin function and promotes differentiation of the transient amplifying population in human prostatic epithelium. *J Cell Sci* 119(7):1416-1424.

8. Yu H-M*, et al.* (2004) Basal prostate epithelial cells stimulate the migration of prostate cancer cells. *Molecular Carcinogenesis* 41(2):85-97.
9. Kurita T*, et al.* (2001) Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. *Cell Death Differ* 2001(8):2.
10. Robinson MJ & Cobb MH (1997) Mitogen-activated protein kinase pathways. *Current Opinion in Cell Biology* 9(2):180-186.
11. Calautti E, Li J, Saoncella S, Brissette JL, & Goetinck PF (2005) Phosphoinositide 3-Kinase Signaling to Akt Promotes Keratinocyte Differentiation Versus Death. *J. Biol. Chem.* 280(38):32856-32865.
12. Evans G & Chandler J (1987) Cell proliferation studies in the rat prostate: II. The effects of castration and androgen-induced regeneration upon basal and secretory cell proliferation. *Prostate* 11:339-351.
13. Mirosevich J*, et al.* (1999) Androgen receptor expression of proliferating basal and luminal cells in adult murine ventral prostate. *J Endocrinol* 162(3):341-350.
14. Wu C-T*, et al.* (2007) Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor. *Proceedings of the National Academy of Sciences* 104(31):12679-12684.

APPENDIX

ABSTRACT

Gordon Research Conference: "Signaling by Adhesion Receptors", South Hadley, MA, June 29-July 4.

Integrin Regulation of Survival in Prostate Tumor Cells Expressing Androgen Receptor

Laura E. Lamb[1, 2], Jelani Zarif[1, 2], and Cindy K. Miranti[1]
[1]Laboratory of Integrin Signaling and Tumorigenesis, Van Andel Research Institute, Grand Rapids, MI
[2]Cell and Molecular Biology Program, Michigan State University, East Lansing, MI

Development of new strategies for more effective treatment of prostate cancer is limited by an incomplete understanding of the mechanisms regulating cell survival of either normal prostate or prostate cancer cells. Androgen receptor (AR) signaling plays an important role regulating cell survival in the prostate and in prostate cancer. Prostate cancer arises from the AR expressing epithelial cells of the prostate. Adhesion to matrix through integrins is required for survival of most epithelial cells. However, AR expressing epithelial cells of the prostate are not adherent to matrix. Paradoxically, in prostate cancer, the epithelial cells expressing AR are adherent to matrix, allowing for the interactions between these two signaling pathways. Our hypothesis is that the interaction of cancer cells with the matrix and the integration of signals from integrins and AR regulate their survival, while AR regulates survival of normal cells independently of integrins. During prostate cancer progression, there is increased expression of the laminin integrin α6β1. Previous studies have demonstrated that AR can lead to increased expression of α6β1, suggesting that AR may drive cell survival by altering integrin expression. We have demonstrated that when plated on laminin, expression of AR in PC3 prostate tumor cells can rescue cells from death induced by inhibition of PI-3K. Expression of AR in PC3 tumor cells leads to increased expression of the pro-survival protein Bcl-x_L and α6β1integrin. Blocking either leads to cell death of the AR expressing cells, suggesting that AR regulates cell survival through enhancement of α6β1/Bcl-x_L signaling pathways in tumor cells. We are currently exploring the mechanism by which AR enhances α6β1/Bcl-x_L signaling. AR expression in PC3 tumor cells also correlates with an increase in number of filopodia per cell, Src activity, and cell migration. In contrast, induction of AR expression in non-tumorigenic primary prostate epithelial cells correlates with loss of integrin expression and reduced adhesion to laminin, resulting in cell death when PI-3K is inhibited.